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14. ABSTRACT

During last year, we have characterized a small molecule inhibitor (MIR214-I) of miR-214. MIR214-I was found to specifically bind to miR-214 in vitro and in vivo. Furthermore, MIR214-I induces programmed cell death and inhibits ovarian cancer stem cell renewal in ovarian cancer cells expressing high levels of miR-214. In addition, MIR214-I selectively reduces tumor growth in xenograft model. Notably, MIR214-I synergizes with cisplatin and overcomes cisplatin resistance.

15. SUBJECT TERMS

microRNA, small molecule inhibitor, ovarian cancer, chemoresistance

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Introduction

Three tasks have been proposed in this project: 1) Identify small molecule inhibitors of miR-19a/b and miR-221/222 using cell-based HTS assay 2) Determine the specificity of miR-19a/b, miR-214 and miR-221/222 inhibitors identified from Aim 1 and 3) Evaluate the small molecule inhibitors of miR-19a/b, miR-214 or miR-221/222 in overcoming ovarian cancer chemoresistance in cell culture and animal mode.

Body:

1. Characterization of miR-214 inhibitor *in vitro*

During last year, we have characterized a miR-214 inhibitor, namely MCCRI (Fig. 1A). We have previously reported that miR-214, miR-155, miR-221/222 and miR-29 target PTEN, FOXO3a, ERα and CDK6 by direct interaction with 3'UTR of the mRNAs, respectively (1-4). To determine MCCRI specificity, luciferase assay was performed in ovarian surface epithelial T80 cells which were transfected with pMIR-3'UTR-PTEN/pre-miR214, pMIR-3'UTR-FOXO3a/pre-miR155, pMIR-3'UTR-ERα/pre-miR221 or pMIR-3'UTR-CDK6/pre-miR29. After incubation for 48 h, cells were treated with and without MCCRI for 6 h and then subjected to luciferase assay. Fig. 1B shows that MCCRI only inhibits miR-214-repressed pMIR-3'UTR-PTEN without significant effects on pMIR-3'UTR-FOXO3a/pre-miR155, pMIR-3'UTR- ERα/pre-miR221 or pMIR-3'UTR-CDK6/pre-miR29, suggesting that MCCRI specifically targets miR-214.

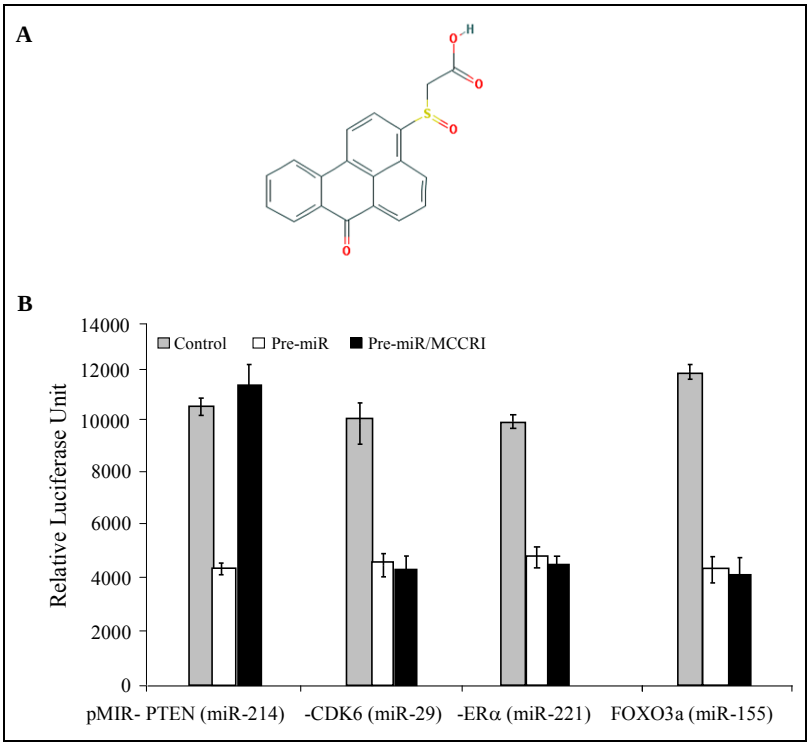

Figure 1. miR-214 inhibitor MCCRI specifically inhibits miR-214. ***(A)*** *Chemical structure of miR-214 inhibitor MCCRI.* ***(B)*** *Luciferase reporter assay. Human immortalized ovarian surface epithelial T80 cells were transfected with pMIR-reporters which contain 3'UTR of PTEN, CDK6, ERα and FOXO3a and corresponding individual pre-miR as well as control oligo and β-gal. After 48 h of incubation, cells were treated with and without MCCRI for 6 h. Luciferase assay was performed and the luciferase activity was normalized to β-gal. Experiments were repeated 3 times in triplicates. Note: MCCRI only abrogated miR-214-repressed pMIR-PTEN reporter activity.*

In addition, MCCRI contains an amino group which allows immobilizing MCCRI on Sepharose beads (GE Healthcare) through covalent linkage using its amino group (5). Thus, we performed *in vitro*

binding assay to determine if MCCRI directly binds to miR-214. NHS-activated Sepharose was equilibrated in DMSO and then incubated with MCCRI and other 2 unrelated compounds (e.g. Akt inhibitors API-1 and API-2), which also contain amino group (5, 6), as controls. Subsequently, the coupled affinity Sepharose beads were incubated with biotin-labeled pre-miR-214. Following washes, the products were eluted, separated on a PAGE gel and then detected. As shown in Fig. 2, pre-miR-214 was only eluted and detected in MCCRI-coupled Sepharose beads, indicating that MCCRI directly binds to miR-214.

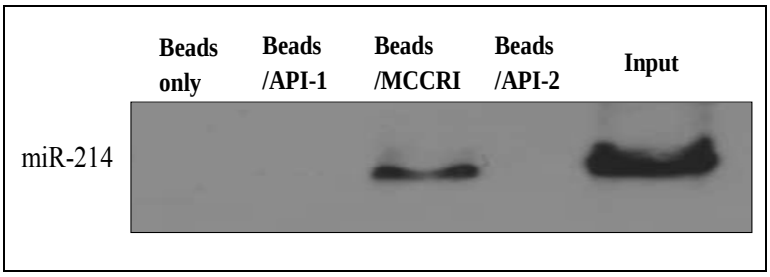

Figure 2. MCCRI directly binds to miR-214. *The indicated compounds were immobilized on NHS-activated Sepharose beads and then incubated with biotin-labeled miR-214. After wash and elution, the eluted products were detected with biotin/streptavidin detection kit.*

Moreover, we have examined the effect of MCCRI on ovarian cancer cell death. Two miR-214-high (A2780CP and OVCAR-8) and two miR-214-low (OV2008 and OVCAR-3) ovarian cancer cell lines were treated with increasing concentration of MCCRI. Cell viability was evaluated after 36 h of treatment. Fig. 3A shows that MCCRI preferentially inhibits the cell survival in miR-214-high cells. Furthermore, Western blot analysis revealed that MCCRI abrogated miR-214-repressed PTEN and p53 expression (Fig. 3B).

Previous studies have shown that miR-214 promotes cell migration and invasion (7, 8). Therefore, we assessed the effects of MCCRI on miR-214-induced cell migration and invasion. Two chamber, with and without matrigel insert, assay revealed that expression of miR-214 promoted ovarian cell migration and invasion which were largely inhibited by MCCRI (Fig. 4).

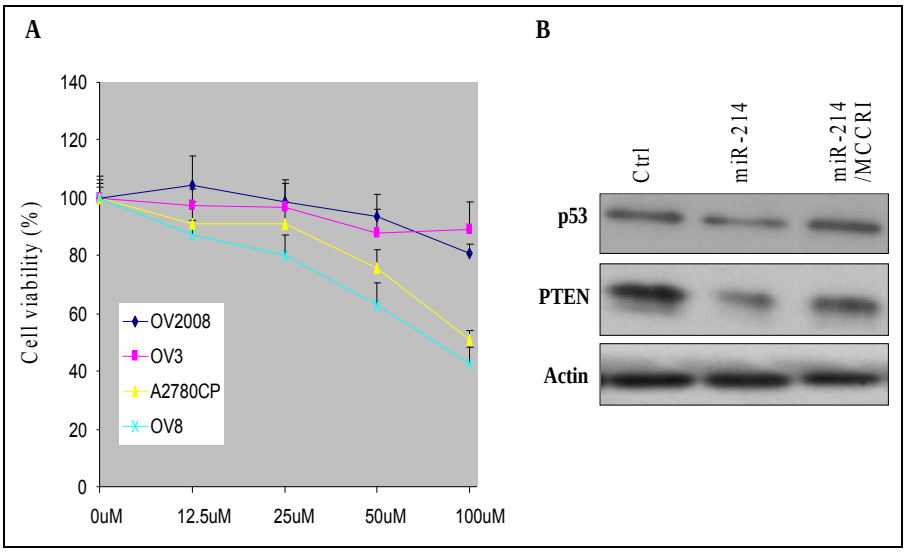

Figure 3. MCCRI selectively inhibits cell survival in miR-214-high ovarian cancer cells. *(**A**) Indicated cells were seeded in 96-well plates and treated with increasing concentrations of MCCRI. After treatment for 36 h, cell viability was evaluated.. (**B**) A2780S cells were transfected with and without pre-miR-214. Following treatment with and without MCCRI, cells were subjected to Western blot analysis with indicated antibodies.*

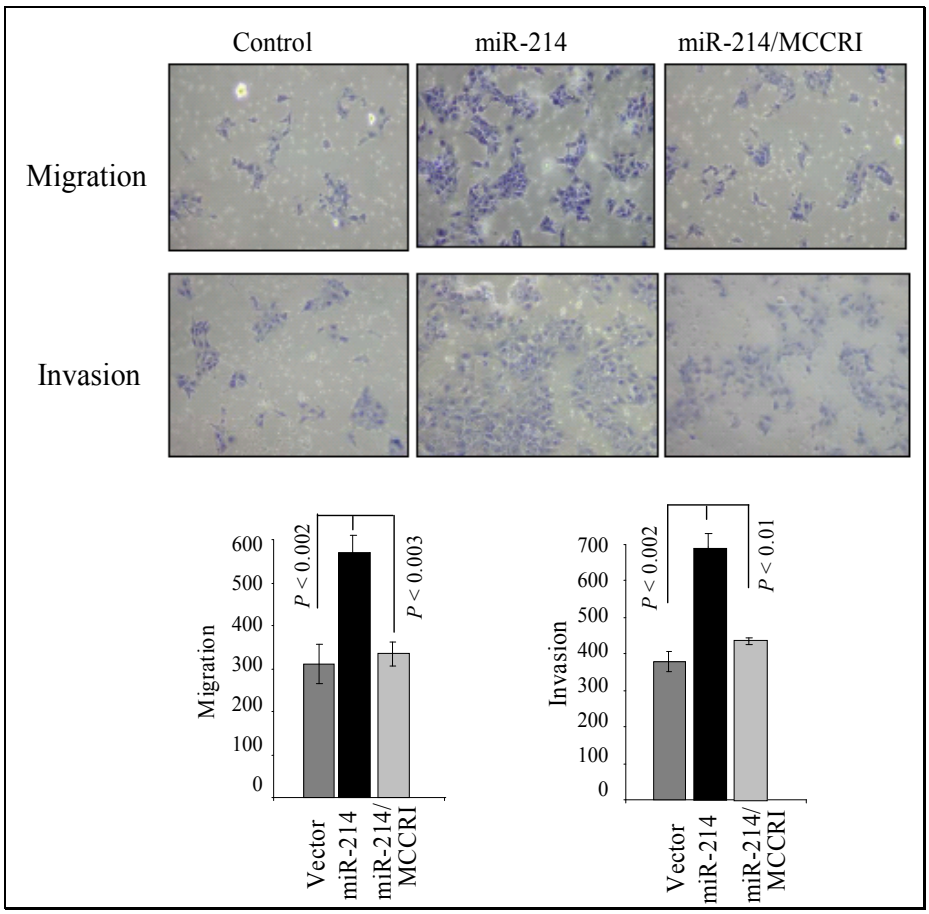

Figure 4. ***MCCRI abrogated miR-214-induced cell migration and invasion.*** *A2780 cells were transfected with pre-miR-214 and control oligo. Following 48 h of incubation, cells were treated with and without MCCRI for 6 h and then subjected cell migration and invasion assay (upper panel). Bottom panels show the quantification.*

2. MCCRI inhibits tumor growth in miR-214-high ovarian cancer cells *in vivo*.

We also examined anti-tumor activity of MCCRI in xenograft model. Briefly, 4 cell lines were subcutaneously injected to nude mice. When the tumor volumes reach approximately 100^3 mm, the mice were divided into 2 groups (10 mice/group). One group was intraperitonealy treated with MCCRI and the other was administered with vehicle control for 27 days. While the anti-tumor activity of MCCRI is moderate, it significantly reduced tumor growth in miR-214-high cell lines (A2780CP and OVCAR-8) when compared to miR-214-low cells (OV2008 and SKOV3; Fig. 5). Western blot analysis also showed that MCCRI induced miR-214 target genes (Fig. 6). In addition, immunohistochemical staining revealed that the proliferation index was reduced in MCCRI-treated tumors (Fig. 7).

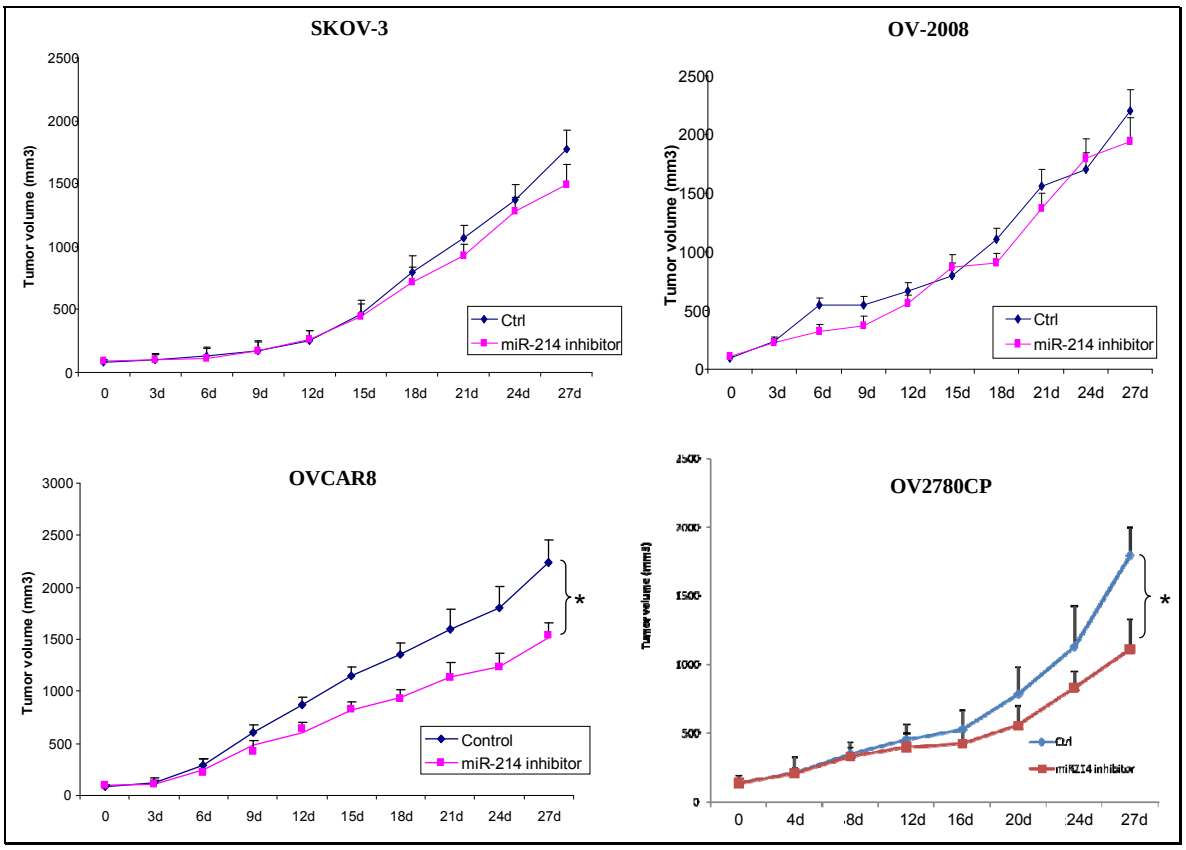

Figure 5*. *MCCRI selectively inhibits tumor growth in miR-214-high ovarian cancer xenograft model. *Indicated cell lines were subcutaneously injected to nude mice (5 x 10^6/mouse). When the tumors reach ~100 mm^3, the mice were intraperitoneally treated with MCCRI at 20 mg/kg (10 mice) or vehicle control daily (10 mice) for 27 days. Tumor growth was monitored and recorded every 3 days.*

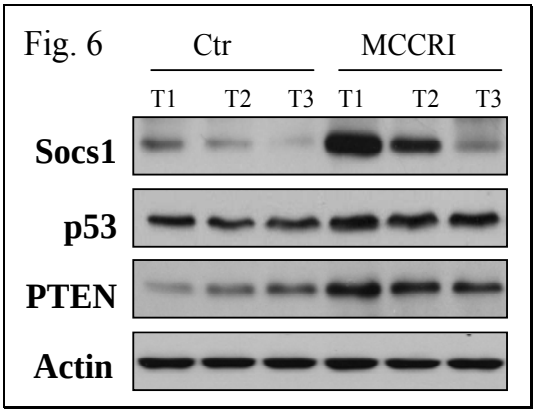

Figure 6. *Western blot analysis of the representative xenograft tumors with indicated antibodies. Note: Socs1, p53 and PTEN are targets of miR-214.*

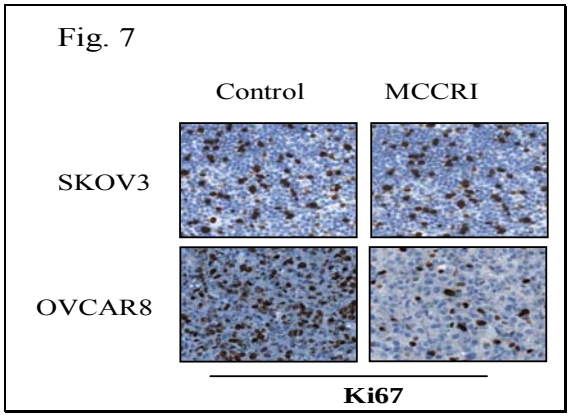

Figure 7. *Ki-67 staining of xenograft tumors from miR-214-low (SKOV3) and miR-214-high (OVCAR8) cells, which were treated with MCCRI and vehicle control.*

3. MCCRI inhibits ovarian cancer stem cell renewal and synergizes with cisplatin to overcome cisplatin resistance.

We have recently reported that miR-214 is a key regulator of ovarian cancer stem cell and plays a critical role in chemoresistance (8). We have also shown that ovarian cancer stem cells express much higher levels of miR-214 than non-cancer stem cells (8). Since cancer stem cells have been shown to contribute to chemoresistance and ovarian cancer relapse, we examined if MCCRI inhibited ovarian cancer stem cell self-renewal. Following treatment with and without MCCRI, C13 cells were labeled with ALDH1 labeling. Flow cytometry analysis showed that MCCRI significantly reduces ALDH1 positive cell (e.g., ovarian cancer stem cell) population (Fig. 8A). Furthermore, stem cell self-renewal assay (9) revealed that sphere formation was inhibited by MCCRI (Fig. 8B). More significantly, we found that MCCRI synergizes with cisplatin and overcomes cisplatin resistance (Fig. 9).

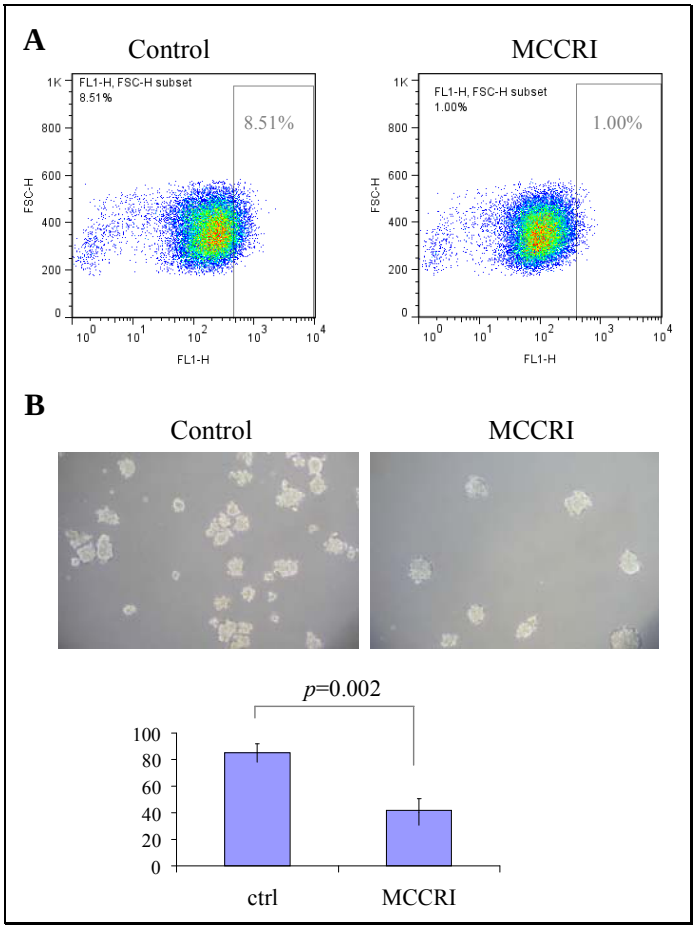

Figure 8. MCCRI inhibits ovarian cancer stem cells. *(**A**) C13 cells were treated with and without MCCRI for 6 h and then were suspended in ALDEFLUOR assay buffer containing an ALDH1 substrate, bodipy-aminoacetaldehyde, at 1.5 μM and incubated for 1 h at 37 °C. Flow cytometry was performed and the data were analyzed FlowJo software (TreeStar). (**B**) Sphere assay. C13 cells were plated in ultra-negative attachment 6-well plates (Corning) at a density of 5000 viable cells/well. Cells were grown in a serum-free sphere culture medium (MammoCult, StemCell Technologies) supplemented with MammoCult proliferation supplements in for 12 days in the presence and absence of MCCRI. Sphere numbers were counted under microscopy.*

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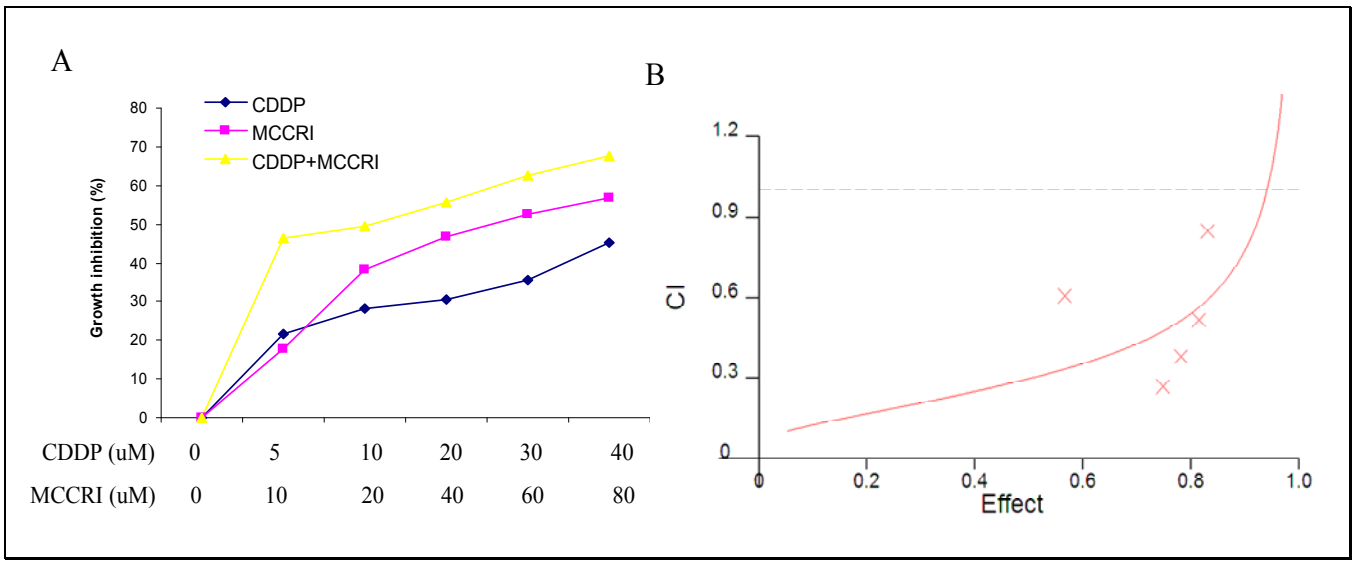

Figure 9. MCCRI synergizes with cisplatin and overcomes cisplatin resistance. *(**A**) Cisplatin-resistance ovarian cancer cell line C13 was treated with cisplatin or MCCRI alone and their combination at indicated concentration. After 72 hour of treatment, cell growth was examined by MTS assay (left). The effects of MCCRI and cisplatin combinations were evaluated with Calcusyn software. CI analysis to determine synergy (defined as CI values < 1) was carried out (right). Each data point is the average of 3 wells each from 3 independent experiments.*

Key Research Accomplishment

1. Identification of a miR-214 specific inhibitor, MCCRI.
2. MCCRI directly binds to miR-214.
3. MCCRI inhibits cell survival and tumor growth in ovarian cancer cell lines that express high levels of miR-214.
4. The properties of ovarian cancer stem cells were inhibited by MCCRI.
5. MCCRI synergizes with CDDP and overcomes CDDP resistance.

Reportable Outcomes

Publication:

1. Xu CX, Tan L, Yang H, Permuth-Wey J, Kruk PA, Wenham RM, Nicosia SV, Lancaster JM, Sellers TA, Cheng JQ. MiR-214 Regulates Ovarian Cancer Cell Stemness by Targeting p53/Nanog. *J Biol Chem* 287:34970-8, 2012.
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Abstract/presentation

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Conclusion

1. Specific miR-214 inhibitor MCCRI has antitumor activity *in vitro* and *in vivo*.
2. MCCRI inhibits ovarian cancer stem cells and overcomes cisplatin resistance.

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Appendices

None